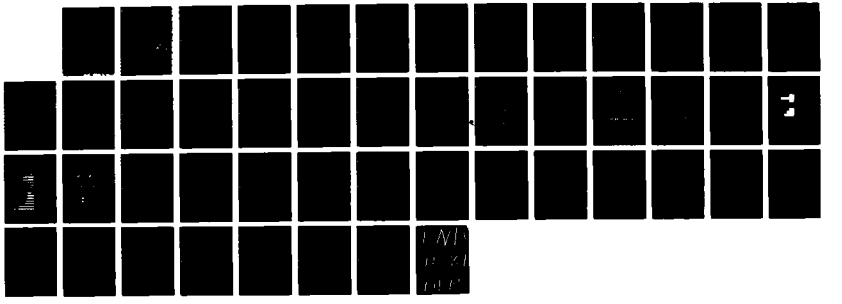
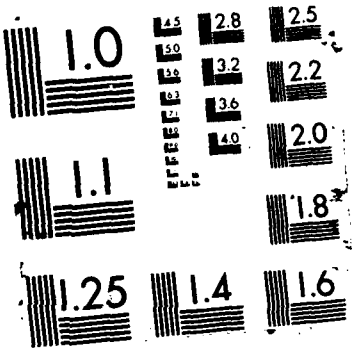
AD-A185 472 INTRINSIC CHOLINERGIC MECHANISMS REGULATING CEREBRAL BLOOD FLOW AS A TARG..(U) CORNELL UNIV MEDICAL COLL NEW YORK LAB OF NEUROBIOLOGY D J REIS ET AL. OCT 85 1/1

UNCLASSIFIED DAMD17-84-C-4185 F/G 6/4 NL




1.0
1.1
1.25
1.4
1.6
2.8
3.2
3.6
4.0
2.5
2.2
2.0
1.8

DTIC FILE COPY

(12)

AD-A185 472

AD _____

INTRINSIC CHOLINERGIC MECHANISMS REGULATING CEREBRAL BLOOD FLOW AS A TARGET FOR ORGANOPHOSPHATE ACTION

Annual Report

Donald J. Reis, Costantino Iadecola, and Stephen P. Arneric

October, 1985

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-84-C-4135

Laboratory of Neurobiology
Cornell University Medical College
New York, New York 10021

DTIC
ELECTE
OCT 2 6 1987
S D
D

Approved for public release; distribution unlimited

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

87 10 19 091

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER	2. GOVT ACCESSION NO. AD-A185 872
3. RECIPIENT'S CATALOG NUMBER	
4. TITLE (and Subtitle) Intrinsic Cholinergic Mechanisms Regulating Cerebral Blood Flow as a Target for Organophosphate Action	5. TYPE OF REPORT & PERIOD COVERED Annual Report 30 Sep 84 - 29 Sep 85
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Donald J. Reis, M.D. Costantino Iadecola, M.D. Stephen P. Arneric, Ph.D.	8. CONTRACT OR GRANT NUMBER(s) DAMD17-84-C-4185
9. PERFORMING ORGANIZATION NAME AND ADDRESS Lab of Neurobiology Cornell University Medical College New York, NY 10021	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research and Development Command Fort Detrick Frederick, MD 21701-5012	12. REPORT DATE October, 1985
	13. NUMBER OF PAGES 44
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)	15. SECURITY CLASS. (of this report) Unclassified
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS: (Continue on reverse side if necessary and identify by block number)

Cerebral blood flow
Cortical acetylcholine release
Muscarinic receptor blockade
Cholinergic vasodilation
Atropine

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The hypothesis of this study is that intrinsic systems in the brain can produce primary vasodilation independent of metabolism, that the system is represented in the fastigial nucleus (FN) of the cerebellum, and that the vasodilation is mediated by the release of acetylcholine (ACh) adjacent to the blood vessels. The cholinergic vasodilation, which is maximum in the cerebral cortex, should be facilitated by organophosphates, the consequence of which will be to abolish local autoregulation, produce an enhanced dependence of the cerebral circulation upon systemic arterial pressure, and thereby render local cerebral vessels vulnerable to breakdown of the blood-brain barrier.

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

20. ABSTRACT (continued)

The two objectives set forth for year 01 of the contract have, in large part, been completed. First, we have replicated the well-established increase in regional cerebral blood flow (rCBF) elicited by FN-stimulation and established that systemic atropine administration completely blocks this cerebrovasodilation. This finding indicates that ACh is released at some link along the neural pathway from the cerebellar FN to the cerebrovasculature. Second, we have demonstrated that the FN-elicited increase in cortical rCBF, an effect that occurs independently of changes in cerebral glucose utilization, is attenuated by local application of atropine to the surface of the parietal cortex. These data suggest that the cortical cerebrovasodilation elicited by FN-stimulation is mediated by the release of ACh at the level of the cerebral cortex. Another essential line of evidence that a cholinergic mechanism in the cerebral cortex is critical for the FN-vasodilation would be to demonstrate that ACh is released locally with FN-stimulation. Our data indicate that the amount of ACh released locally in the parietal cortex following FN-stimulation is too small to be detected by the currently available assay techniques.

DTIC
COPY
INSPECTED

Accession For
NTIS CRA&I
DTIC TAB
Unannounced
Justification
By
Distribution/
Availability Codes
Dist | Avail and/or Special
A-1

AD _____

INTRINSIC CHOLINERGIC MECHANISMS REGULATING CEREBRAL BLOOD FLOW AS A TARGET FOR ORGANOPHOSPHATE ACTION

Annual Report

Donald J. Reis, Costantino Iadecola, and Stephen P. Arneric

October, 1985

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-84-C-4185

Laboratory of Neurobiology
Cornell University Medical College
New York, New York 10021

Approved for public release; distribution unlimited

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

SUMMARY

The hypothesis of this study is that intrinsic systems in the brain can produce primary vasodilation independent of metabolism, that the system is represented in the fastigial nucleus (FN) of the cerebellum, and that the vasodilation is mediated by the release of acetylcholine (ACh) adjacent to the blood vessels. The cholinergic vasodilation, which is maximum in the cerebral cortex, should be facilitated by organophosphates, the consequence of which will be to abolish local autoregulation, produce an enhanced dependence of the cerebral circulation upon systemic arterial pressure, and thereby render local cerebral vessels vulnerable to breakdown of the blood-brain barrier.

The two objectives set forth for year 01 of the contract have, in large part, been completed. First, we have replicated the well-established increase in regional cerebral blood flow (rCBF) elicited by FN-stimulation and established that systemic atropine administration completely blocks this cerebrovasodilation. This finding indicates that ACh is released at some link along the neural pathway from the cerebellar FN to the cerebrovasculature. Second, we have demonstrated that the FN-elicited increase in cortical rCBF, an effect that occurs independently of changes in cerebral glucose utilization, is attenuated by local application of atropine to the surface of the parietal cortex. These data suggest that the cortical cerebrovasodilation elicited by FN-stimulation is mediated by the release of ACh at the level of the cerebral cortex. Another essential line of evidence that a cholinergic mechanism in the cerebral cortex is critical for the FN-vasodilation would be to demonstrate that ACh is released locally with FN-stimulation. Our data indicate that the amount of ACh released locally in the parietal cortex following FN-stimulation is too small to be detected by the currently available assay techniques.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

A. Background

That acetylcholine (ACh) may in fact play a role in control of the cerebral circulation has long been surmised (1, 2, 3). To summarize, there is abundant pharmacological evidence that cerebral vessels will dilate in response to topical or intraarterial administration of ACh (4), often without change in metabolism, that cerebral vessels contain ACh receptors, and that nerves innervating cerebral vessels, including those arising from branches of the VIIth cranial nerve (specifically, the greater superficial petrosal nerve), will produce vasodilation that is blocked by atropine is further evidence for an extrinsic cholinergic regulation.

Whether intrinsic cholinergic pathways contribute to the control of cerebral blood flow (CBF) and metabolism is less certain. Stimulation of systems in brain which will produce local increases in brain metabolism and blood flow in man, for example, the classical sensory pathways, will release ACh from cortical islands (5). Moreover, the fact that neostigmine will facilitate the vasodilation associated with cortical arousal or hypercapnia, an effect blocked by atropine (see Scremin 1982, for review) and that the effect is independent upon metabolism suggests that intrinsic systems in the brain play a role. The identity of the neural pathways mediating these responses is totally unknown.

Over the past several years, our laboratory has been investigating the mechanisms by which the brain regulates its own circulation (3). Our approach has been to examine the effects of electrical stimulation of anatomically specific sites in rat brain on regional cerebral blood flow (rCBF) or metabolism, the latter measured by estimating regional glucose utilization (rCGU). By sampling changes in rCBF in anesthetized animals which are paralyzed and ventilated (thereby maintaining blood gases constant) and by maintaining arterial pressure in the autoregulated range any evoked changes in flow can be attributed to excitation of specific pathways. By analyzing this distribution of flow changes anatomically it is possible to assess if evoked changes are regional and adhere to specific neural pathways or are more widespread. By comparing the distribution and change in rCBF with rCGU we can determine if changes in flow are "coupled" or "uncoupled" from metabolism. Finally, using pharmacological probes we can begin to approach the neurochemistry of these networks as well. Using this strategy, we have discovered three parallel systems in the brain which act to modify rCBF and rCGU in the cerebral cortex in totally different ways (3).

One of these systems is organized in the cerebellar fastigial nucleus (FN) and will, when excited, increase rCBF throughout the brain. The greatest increases are in the cerebral cortex, where rCBF increases up to 2.5-fold. The increase in cortical rCBF is unassociated with changes in metabolism (6) and is associated with the abolition of cerebrovascular autoregulation (7); the response is mediated by as yet undefined pathways from cerebellum to cerebral cortex (8). The fact that this cerebrovascular vasodilation is not abolished by spinal cord transection, sympathectomy, or section of the VIIth cranial nerve (9, 6, 7), indicates that the response is mediated by intrinsic neural pathways. These studies have revealed a heretofore unrecognized intrinsic neural system in the brain through which rCBF can be modified independently of cerebral metabolism.

In preliminary experiments we have observed that the global cerebral vasodilation elicited from FN is abolished by atropine (10). This indicates that the effect is mediated by the release of ACh somewhere in the brain. The question remains, At what site in the brain does atropine act to block the cerebrovascular vasodilation elicited from FN? The pathways by which impulses from FN are relayed to the cerebral cortex are unknown. Atropine could be blocking a cholinergic link along a subcortical pathway. Conversely, it could be acting to block the actions of ACh released within the cortex itself, either from penetrating cholinergic fibers arising from the basal forebrain (11), by acting upon local cholinergic interneurons in the cortex (12), or even by acting upon endothelial cells to release substances directly into the microenvironment of the brain (13).

B. Working Hypothesis

The hypothesis of this study is that intrinsic systems in the brain can produce primary vasodilation independent of metabolism, that the system is represented in the FN of the cerebellum, and that the vasodilation is mediated by the release of ACh adjacent to the blood vessels. The cholinergic vasodilation, which is maximum in the cerebral cortex, should be facilitated by organophosphates, the consequence of which will be to abolish local autoregulation, produce an enhanced dependence of the cerebral circulation upon systemic arterial pressure, and thereby render local cerebral vessels vulnerable to breakdown of the blood-brain barrier. In the face of elevations of arterial pressure which, under normal circumstances, will be counterbalanced by the autoregulatory events, organophosphate exposure might lead to vulnerability to cerebral edema, hemorrhage or ischemia.

C. Specific Aims (Year 01)

As outlined in the contract, the specific aims for the experiments performed during year 01 are:

1. To establish that the cortical cerebrovasodilation elicited by electrical stimulation of the FN of the cerebellum is mediated by ACh.

2. To determine whether the cholinergic link is at the level of the cerebral cortex and, if so, whether ACh is released from the cerebral cortex during FN-stimulation.

METHODS

A. General Procedures

Male Sprague-Dawley rats, weighing 290-380 g, were maintained in a thermally controlled (26-27°C), light-cycled (0700 on-1900 off) environment, and given chow and water ad lib. Rats were anesthetized with alpha-chloralose (40 mg/kg, s.c.) after induction with halothane (2.5% in 100% O_2) blown over the nose. Thin wall vinyl (o.d. = 0.5 mm) and polyethylene (o.d. = 1.3 mm) catheters were placed in each femoral artery and vein, respectively, and the trachea was cannulated.

Animals were then placed in a stereotaxic frame with the head adjusted so that the floor of the IVth ventricle was horizontal (bite bar position: -11 mm). After connecting the tracheal cannula to a small-animal respirator (Harvard Apparatus, 680), the animals were paralyzed with tubocurarine (0.5 mg/kg, i.m., initially; supplemented with 0.2 mg/kg hourly), and ventilated (80 cpm) with 100% O_2. Halothane was continued at a reduced rate (1%) during surgery. Continuous monitoring of arterial blood pressure and heart rate was done through one of the arterial catheters connected to a Statham P23Db transducer, which was coupled to a chart recorder.

The lower brainstem and caudal half of the cerebellum were exposed by an occipital craniotomy as described in detail previously (9). After completion of the surgery, halothane was discontinued. A small volume (about 0.2 ml) of arterial blood was sampled after surgery for measurement of pO_2, pCO_2 and pH by a blood gas analyzer (Instrument Laboratories, Model Micro 13). Arterial blood gases were maintained so that pO_2 was 100 mmHg; pCO_2 = 33-38 mm Hg; and pH = 7.35-7.45. Adjustments were made by changing the stroke volume of the ventilator.

B. Electrical Stimulation of FN

The FN was stimulated with cathodal current delivered through monopolar electrodes fabricated from Teflon-insulated stainless steel wire (150 um, o.d.), carried in 28-gauge stainless steel tubing, and exposed at the tip for 100 um. The anode (ground) was a clip attached s.c. to neck muscle. Electrical pulses were generated by a square-wave stimulator (Grass, S-88) and constant current was passed through a photoelectric stimulus isolation unit (Grass model PS1U6). The stimulus current intensity was determined by continuously displaying on an oscilloscope the voltage drop across a 10 ohm resistor.

The electrode was mounted on a stereotaxic micromanipulator and lowered into the cerebellum with a posterior inclination of 10°. Localizing the FN pressor response (FNPR) was used as a means to identify the active site which elicits increases in CBF. The area of the cerebellum explored extended 4.8-5.2 mm anterior to, 0.6-1.0 mm lateral to, and 2.0-0.5 mm above the calamus scriptorius, the stereotaxic reference point. To localize the most active area of the FN, the electrode was moved in steps of 0.5 mm while stimulating with 8 sec trains of 0.5 ms duration pulses, at a frequency of 50 Hz and an intensity of 20 uA. When the FNPR was elicited, the threshold current, defined as the stimulus current which increases arterial pressure 10 mmHg, was determined. For blood flow and release experiments, the stimulus current was set at five times threshold.

C. Cerebral Blood Flow Measurement

CBF was measured using ^{14}C-iodoantipyrine (IAP) as indicator (14). Tissue concentrations of IAP were obtained by the tissue sampling technique or autoradiographically. The brain:blood partition coefficient used was 0.8(14).

4-(N-methyl-^{14}C) IAP in ethanol (NEN, 40-60 mCi/mmol) was dissolved in about 1 ml of normal saline after elimination of ethanol. Animals received 2000 units of heparin i.v. approximately 10 min prior to indicator infusion. The indicator was infused (5 uCi/100 g of body weight) at a constant rate over 30-35 sec through the femoral venous

catheters by an infusion pump (Harvard Apparatus Model 940). Simultaneously, about 50 ul of arterial blood was sampled every 2-5 sec through the femoral arterial catheter in order to obtain the arterial concentration-time curve of IAP. The sampling catheter was kept short (5 cm) to more accurately reflect the arterial concentration-time curve of IAP. Aliquots (40 ul) of arterial blood were transferred to scintillation vials filled with 1 ml tissue solubilizer (NEN: Protosol, ethanol, 1:1 v/v). The blood mixture was incubated at 60°C for 1 hour, decolorized with 30% hydrogen peroxide, and mixed with 15 ml of Biofluor (NEN). Radioactivity was measured by a liquid scintillation spectrophotometer (Beckman, LS 5801) and corrected to disintegrations/min (d.p.m.), using an external standard.

1. Measurement of tissue concentration of IAP by regional dissection. Approximately 30 sec following the start of the infusion of IAP, the animal was killed by a rabid bolus injection of 1 ml of saturated KCl in the femoral vein catheter. The brain was rapidly removed from the skull, briefly (10 sec) placed in liquid Freon (-30°C), and put on an ice-cold glass plate. Right and left samples of 5 brain regions were dissected: parietal cortex (beneath, medial and lateral to the cortical pipette); frontal cortex (2-4mm rostral to the pipette); occipital cortex (2-4mm caudal to the pipette); caudate nucleus and hippocampus. Tissue samples were transferred to tared scintillation vials and the tissue weights determined. After solubilization of the tissue by Protosol (1 ml), 10 ml of scintillation cocktail (Econofluor, NEN) was added and the samples counted.

2. CBF by Autoradiography. Thirty seconds after the start of the infusion, the animal was killed by i.v. injection of a bolus of saturated KCl. The brain was rapidly removed from the skull, frozen in liquid Freon at about -30°C, coated with embedding material (Lipshaw), and mounted on a cryostat chuck. The brain was allowed to equilibrate for at least 3 hours at -20 to -22°C in the cryostat.

Three consecutive sections, precisely 20 um thick, were cut on an International E. cryostat at -20°C and collected at 300 um intervals. Sections were removed from the knife, using coverslips at room temperature, and immediately dried on a hot plate at 60°C. Coverslips were mounted on glass slides and sequentially fitted into an X-ray cassette, together with a set of (^{14}C)methyl-methacrylate standards (Amersham). Sections and calibration standards were exposed on X-ray film (Kodak SB-5) for 1 week. X-ray films were removed and manually developed according to the instructions supplied with the film. Alternate sections were stained with cresyl violet and mounted on microscope slides.

3. Analysis of the autoradiograms. The analysis of autoradiograms was performed using a computerized image analysis system (Spatial Data System, Eyecom II; PDP 11/45). The autoradiograms, representing brain sections and standards, were digitized from the film placed on a light box, using a Vidicon scanner. A calibration curve was obtained by relating optical density (O.D.) to ^{14}C concentration (nCi/g).

For each structure, bilateral readings were taken over at least 3, usually 4, consecutive autoradiograms. Replicate O.D. measurements of the same brain structure in 4 consecutive sections showed, for the 27 areas analyzed, an average variation coefficient of 6.3%, ranging from 1.6% to 11%.

4. Calculation of CBF. CBF (ml/100 g x min) was calculated by obtaining a relationship between CBF and tissue concentration of IAP, using a computerized approximation of the equation developed by Kety (14). Details on the accuracy of the computer program and on the resolution of the method at high CBF values have been published in an earlier report (9).

D. Cortical Superfusion Device

A schematic of the device used to apply atropine to the cortical surface and collect superfusate during the release experiments is shown in Fig I. For the placement of this device, small holes (2.5 mm,o.d.) were drilled bilaterally over the parietal cortex in an area 2.0-4.5 mm lateral and $^{+}0.5$ to -2.0 mm anterior-posterior to bregma. Following reflection of dura, the device was stereotaxically positioned on this standardized region of the intact sensory motor cortex. Microscopic examination of the pipette placement was used to avoid occlusion of pial vessels. The cortical surface and superfusate temperature were clamped at 37 $\pm$ 0.5°C with the aid of a surface thermistor and overhead heating lamp coupled through a servo-mechanism (YSI Instruments, model 73A). Solutions filling the device and contacting the cortex were bubbled with 95% O_2: 5% CO_2 immediately before each experiment to carefully control for pH (7.3-7.4), pCO_2 (30-40 mmHg) and pO_2 (300-500 mmHg), then superfused over the pial surface at 10 ul/min with an infusion pump (Harvard Apparatus model 940). The superfusate consisted of a modified Kreb's-bicarbonate buffer containing physostigmine to inhibit ACh degradation (in mM: NaCl, 118; $CaCl_2$, 1.2; KCl, 4.8; $MgSO_4$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 25; choline chloride, 0.001; physostigmine, 0.1). The area of the cortical surface exposed was 0.018 cm^2.

E. Electrocorticography

Electrocorticograms (ECoG's) were recorded ipsilaterally to the side of the stimulus presented (e.g., FN-stimulation). The ECoG was recorded bipolarly between a pair of stainless steel Teflon-coated wires fixed to the outside of the superfusion device. The electrodes contacted the dura firmly, while ground was a metal clip placed s.c. in the neck. The ECoG signal was fed into an AC amplifier (Grass, Model 7P511) and displayed on the polygraph.

F. Experimental Protocol In Vivo

One hour after completion of surgery, a stimulating electrode was lowered through the cerebellum and positioned in the most "active" portion of the FN, as described above. Care was taken during exploration always to avoid large, abrupt changes in arterial pressure (AP) and to maintain AP within the autoregulated range of CBF for the rat (80-150 mmHg). Others have shown that passive vasodilation as a consequence of mechanical distortion may occur and may produce artifactual results if these precautions are not adhered to (15). The electrode was left in place at the active site, and blood gases were carefully adjusted. At this point in the protocol, the animal was either prepared for cortical application of drugs and measurement of rCBF or prepared for measuring the release of ^{3}H-ACh.

1. Atropine Application/rCBF Measurement. In the experiments in which atropine was applied to the cerebral cortex the cortical superfusion device was carefully placed on the pial surface 30 min prior to rCBF measurement. Either vehicle (Kreb's-bicarbonate buffer) or atropine sulfate (100 uM), was superfused for 10 min prior to electrical stimulation of FN. In other experiments, either atropine or methylatropine as administered systemically (i.v.) in adrenalectomized animals 10 min. prior to the onset of electrical stimulation of FN. For rCBF measurement, the FN was stimulated with an intermittent stimulus train (1 sec on/1 sec off; pulse duration, 0.5 msec; frequency, 50 Hz). During the first 2-4 min the intensity of the stimulus was gradually increased to reach 5x the threshold current, i.e., the current which elicited a 10-20 mmHg elevation of mean arterial pressure (MAP), and at the same time the evoked rise in AP was gradually reduced by slow, controlled withdrawal of blood (4-6 ml). The controlled hemorrhage maintained the AP in the autoregulated range. FN-stimulation continued for 7-10 min, during which the AP remained stable and the blood gases were finally adjusted.

At the end of this phase, ^{14}C-IAP was infused, the animals were killed 30 sec later and the amount of radioactivity in the arterial blood and brain tissue was determined.

2. Release of ^{3}H-ACh From Cortical Surface. For these studies, a stimulating electrode was positioned in the FN and the superfusion device placed shortly after completion of the surgery. Just prior to the placement of the device, nerve terminal stores of the cholinergic innervation to the primary motor cortex were prelabeled with ^{3}H-methylcholine (1.5 uCi/1 ul, specific activity 80 uCi/nmol, New England Nuclear). The ^{3}H-methylcholine was microinjected at a depth of 1.5 mm below dura through a 70 um (o.d.) glass pipette at a rate of 200 nl/min with the aid of a micromanipulator and air-driven mechanical valve system (Amaral and Price, 1983). When the cortical superfusion device was put in place, the pial surface was superfused with modified Kreb's-bicarbonate buffer (containing 100 uM physostigmine) at a rate of 10 ul/min. Superfusate was collected in 8 min epochs for 2 hours into microcentrifuge tubes containing 8 ul 1.0 N perchloric acid. Immediately following the experiment, the samples were frozen (-20°C) for later analysis of ^{3}H-ACh (see below for details).

Release of ^{3}H-ACh was quantified during three paradigms: 1) spontaneous, basal release; 2) electrical stimulation of FN; and 3) depolarization of the cortical surface with 55 mM K^{+}. Previous in vitro studies indicated that 55 mM K^{+} is a supramaximal stimulus to elicit the maximal release of ^{3}H-ACh from cerebral cortex slices. Increased potassium concentrations were compensated for by an equiosmolar decrease in sodium concentration.

Preliminary experiments (N=5) demonstrated that 1 hour following microinjection of ^{3}H-methylcholine, more than 99% of the tritium was restricted to the right parietal cortex (Table I). This suggested that the release of ^{3}H-ACh measured during the experiments was not from adjacent structures.

G. ^{3}H-ACh Release From Brain Slices

Rats were killed by decapitation. The brains were rapidly removed and placed in ice-cold Kreb's-bicarbonate buffer containing 100 um physostigmine. Slices of cerebral cortex and underlying caudate nucleus were obtained from tissue dissected 1.5 mm rostral-0.5 mm caudal to the anterior commissure. Coronal sections (0.3-0.5 mm thick) were prepared with a McIlwain tissue chopper and bisected into right and left halves. Up to 8 hemi-sections of caudate nucleus or cortex were prepared from each brain. To correct for possible regional variations in release, all of the samples of caudate nucleus or cortex from one brain were pooled, and 2 slices were randomly assigned to either control (vehicle) or peptide treatment. Average wet tissue weights for the slices were 18 $\pm$ 3 mg for cortex slices (N=25) and 23 $\pm$ 5 mg for caudate nucleus slices (N=25).

Release of ^{3}H-ACh was analyzed using modifications of the radiochemical method described by Hadhazy and Szerb (16). Briefly, 2 slices were incubated for 20 min at 37°C in Kreb's-bicarbonate buffer gassed with 95% O_2: 5% CO_2, which also contained 20 uCi/ml of ^{3}H-methylcholine (New England Nuclear, 80 Ci/nmol). The slices were then transferred at 5 min intervals through a series of 1.5 ml microcentrifuge tubes (Eppendorf) containing 1.0 ml of gassed Kreb's-bicarbonate buffer to which 10 uM bacitracin was added to inhibit peptide degradation. Neurotransmitter release was evoked by exposing the tissue to potassium (5-55 mM) for 5 min. Increased potassium concentrations were compensated by an equiosmolar decrease in sodium concentration. To determine the Ca^{2+} dependency of neurotransmitter release, the Kreb's-bicarbonate solution was modified by replacing Ca^{2+} with Mg^{2+} (1.2 mM) 10 min prior to, and during, potassium depolarization. Immediately following exposure of the brain slices to the buffer, the buffer was stored at -20°C for later analysis of ^{3}H-ACh.

H. Biochemical Assay of ^{3}H-ACh

Since the ACh stores were labelled with ^{3}H-methylcholine, the radioactivity released from the cerebral cortex was separated by several techniques to quantify the ^{3}H-ACh released.

^{3}H-Choline was separated routinely from ^{3}H-ACh by an enzymatic liquid-cation exchange method modified from Briggs and Cooper (17). Aliquots (40 ul) of the superfusate were incubated in a final volume of 200 ul containing 100 mM NaH_2PO_4 buffer (pH=8.5), 0.36 mM ATP, 6.0 mM $MgCl_2$, and 50 ug/ml of choline kinase for 30 min at 37°C. The reaction was stopped by placing the sample on ice, adding 0.5 ml of tetraphenylboron in 2-heptanone (10 mg/ml) and shaking vigorously. The samples were centrifuged, the organic layer containing ^{3}H-ACh was removed and the extraction was repeated with another 0.5 ml of tetraphenylboron in 2-heptanone. The radioactivity in both the organic and aqueous layers was determined by standard liquid scintillation counting methods. Using ^{3}H-choline and ^{14}C-ACh standards (80 uCi/nmol, New England Nuclear), 99% of the choline kinase-treated ^{3}H-choline (10-10,000 pmol) was converted to ^{3}H-phosphorylcholine, which remained in the aqueous layer, and 98% of the ^{14}C-ACh (up to 1 mg/ml) was extracted into the organic layer. When the radioactive substance extracted into the organic layer was lyophilized, reconstituted, treated with acetylcholinesterase (100 U/ml), and separated as described above, 99% of the substance was found in the aqueous layer, thereby indicating that the substance was ACh.

Further confirmation of the identity of the separated radioactivity was accomplished by the thin layer chromatographic method described by Richter and Marchbanks (18). The incubation mixture was lyophilized and the compounds reconstituted in a small volume (10 ul) of distilled water containing carrier ACh, choline and phosphorylcholine (3 mg/ml each). The mixture was separated on cellulose plates without $CaSO_4$ binder, using butanol:ethanol:acetic acid:water (8:2:1:3 v/v), as the solvent system. The spots were visualized using Dragendorf's reagent, scraped into scintillation counting vials, eluted off the cellulose with 1.0ml of H_2O: ethanol (1:1 v/v)and counted. Typical Rfs that were obtained with this method were:ACh, 0.70; choline, 0.57; and phosphorylcholine, 0.26.

J. Calculations and Statistics

Data were expressed as the mean $\pm$ S.E.M. Routinely, data were expressed as a percent of the control (vehicle) response for ease of graphical presentation. However, the statistical analyses were always performed on the absolute values of the data obtained from each experimental group. Data were analyzed by analysis of variance (ANOVA) with treatment differences being detected by Duncan's new multiple-range test. The criterion of statistical significance was $p \quad 0.05$.

Release values for in vivo experiments were expressed as the amount of neurotransmitter released per cm^2. For the in vivo release experiments, basal release was defined as the average of the two epochs (br_1, br_2) measured immediately prior to the two epochs during the evoked release (Er_1, Er_2). An estimate of the predicted spontaneous release (Sr) was determined during the period of interest as a linear interpolation between the basal release

(i.e., $\frac{br_1 + br_2}{2}$) and the average of the two poststimulus release samples

(i.e., $\frac{pr_1 + pr_2}{2}$). Thus spontaneous release was

$Sr = \frac{br_1 + br_2}{4} + \frac{pr_1 + pr_2}{4}$, while the

evoked release was $\frac{Er_1 + Er_2}{2}$. For the given experimental groups, the mean $\pm$ S.E.M. of Sr and Er were calculated and compared by ANOVA.

In some in vitro experiments, the percent fractional rate of ^{3}H-ACh outflow (d.p.m.'s of tritium)/5 min was calculated as: (d.p.m.'s tritium outflow per 5 min)/(d.p.m.'s tritium in the slice at the start of the respective 5 min period) x 100. The amount of radioactivity remaining in the slice at the end of the experiment was determined by dissolving the tissue in 1.0 ml of protosol and counting the tritium by standard liquid scintillation techniques. Data were expressed as a percent of the tritium content of the slice at the respective fraction examined.

RESULTS

FN-elicited Increases in CBF

Electrical stimulation of the FN elicited the well-established elevation in AP and heart rate (HR) (19) (Table II). It also elicited EEG changes characterized by stimulus-locked, regular, slow-wave activity (2-3 Hz), which on occasion persisted for 2-4 sec after termination of the stimulus (Fig. IIA).

In untreated rats (N=5), FN-stimulation elicited a global increase in rCBF (Table III). During this time body temperature (not shown) and arterial blood gases (Table IV) were monitored and maintained within physiological ranges. The adrenal glands were bilaterally removed to minimize the amount of blood withdrawn during stimulation to keep AP in the autoregulated range. The blood flow increases ranged from 121 $\pm$ 7% of control in hypothalamus to 246 $\pm$ 50% in frontal cortex ($p < 0.05$, Table III), and were similar in magnitude and regional pattern to those previously reported from our laboratory (6, 9). In the inferior colliculus, the 122% of control increase did not reach statistical significance ($p > 0.05$).

When local cerebral blood flow (lCBF) was measured autoradiographically, similar values were obtained for resting and FN-elicited increases in blood flow when compared to those values obtained from corresponding areas using the regional dissection technique (Table V). These control experiments were the preliminary phase of the experiments designed to examine the effects on lCBF of cholinergic agents and nerve agents microinjected into the cortex.

Effect of Systemic Atropine on the FN-elicited Increase in CBF

Atropine (0.3 mg/kg, i.v.) did not significantly change the minimal current required for elevation of AP (i.e., threshold current) nor the magnitude of AP elevations elicited from FN stimulation at 5 times threshold (Table II). Basal HR rose by 75 $\pm$ 11 beats per minute (bpm) (mean $\pm$ S.E.M.) ($p < 0.001$) after atropine, while the increase in HR elicited by FN-stimulation was significantly reduced (Table II). Atropine did not affect basal EEG nor the evoked change in EEG activity described above (Fig. II). Resting rCBF in unstimulated rats was not affected by atropine ($p > 0.05$; $N = 5$); however, the drug virtually abolished the increase in rCBF elicited by FN-stimulation (Table III; N=5). Methylatropine similarly abolished the FN-elicited increase in rCBF (Table VI). Adrenalectomy did not affect rCBF and only minimally modified the increase elicited by FN-stimulation (Table VI).

Effect of Local Application of Atropine on the Cortical Cerebrovasodilation Elicited by FN-Stimulation

Resting rCBF did not differ between sides of the frontal, parietal and occipital cortices, caudate nucleus or hippocampus (Table VII). Application of the pipettes to the parietal cortex did not alter resting rCBF in the parietal cortex or subcortical regions (Table VII). During these experiments arterial blood gases were monitored and maintained within physiological ranges (Table VIII). The pH and gases of the cortical superfusates were also carefully controlled (Table IX). FN-stimulation increased rCBF in all regions (Table X) with increases ranging from 171% of control in hippocampus to 219% in parietal cortex. Placement of the cortical superfusion device did not alter the ECoG pattern observed following FN-stimulation, (Fig. IIIA), nor did it affect the ability of the cortex to desynchronize in response to electrical stimulation of the pontine reticular formation (Fig. IIIB).

In 5 animals atropine sulfate (100 uM) was applied on the right parietal cortex 20 min prior to FN-stimulation, while vehicle ws applied on the left. Atropine did not alter resting cortical rCBF but significantly reduced by 55% the FN-elicited increase in rCBF in the ipsilaterial, but not contralateral, fronto-parietal cortex (Table X). Adjacent structures were not affected ($p > 0.05$, N=5).

In contrast, the application of atropine sulfate did not effect the cortical cerebrovasodilation elicited by hypercarbia (Table X), since elevating arterial pCO_2 to 59.0 ± 1.4 mmHg increased rCBF in all regions, including the area treated with atropine, and to a magnitude similar to that of FN-stimulation (e.g., parietal cortex rCBF = 170 + 27 ml/100 g x min, N=5). These results indicate that the blockade by atropine of the FN-elicited vasodilation was not the result of non-specific vasoparalysis.

^{3}H-ACh Release

In vitro. The basal and potassium-evoked release of ^{3}H-ACh from slices of cerebral cortex and caudate nucleus was analyzed in the presence and absence of extracellular Ca^{2+}. After the initial preincubation with ^{3}H-choline, there was a decline in tritium, followed, within 10-20 min, by achievement of a steady-state basal efflux (Fig. IV). The basal fractional release was not altered in either the caudate nucleus or the cortex by removing extracellular Ca^{2+} (Table XI). The rate of basal tritium efflux was 176% greater in cortex than in caudate nucleus (Table XI).

Depolarization with 35 mM K^+ in the presence of Ca^{2+} elicited a marked rise ($p < 0.05$) in the fractional rate of tritium released from slices of cortex and caudate nucleus (Table XI). This release was stimulus-locked (Fig. IV). The evoked efflux of tritium was dependent upon the concentration of potassium over a range of 15-35 mM and was maximal at 45 mM K^+ (Fig. V). The maximal K^+-evoked release of ^{3}H-ACh was approximately fourfold greater in caudate nucleus as compared with cortex (Fig. V), probably reflecting the difference in the cholinergic innervation of these two brain regions.

These data demonstrated that we could measure small quantities of ^{3}H-ACh released from the cortex and that the release occurred according to established criteria (i.e., release was Ca^{2+}-dependent, stimulus-locked and dependent on depolarization stimulus). Also it established the depolarization stimulus required to evoke the maximal release of ^{3}H-ACh from the cortex.

In vivo. Figure IV illustrates a representative experiment showing the effects of electrical stimulation of the FN and of potassium depolarization on the release of ^{3}H-ACh from the parietal cortex of anesthetized rat. Within 30 min of cortical superfusion, the rate of release of ^{3}H-ACh stabilized. In 4 animals, electrical stimulation of the FN did not alter the spontaneous release of ^{3}H-ACh (Fig. VII). In contrast, maximal depolarization of the cortex by 55 mM K^+ elicited a 241% increase in the release of ^{3}H-ACh. The magnitude of the in vivo K^+ evoked release of ^{3}H-ACh was similar to that demonstrated in vitro (Fig. V).

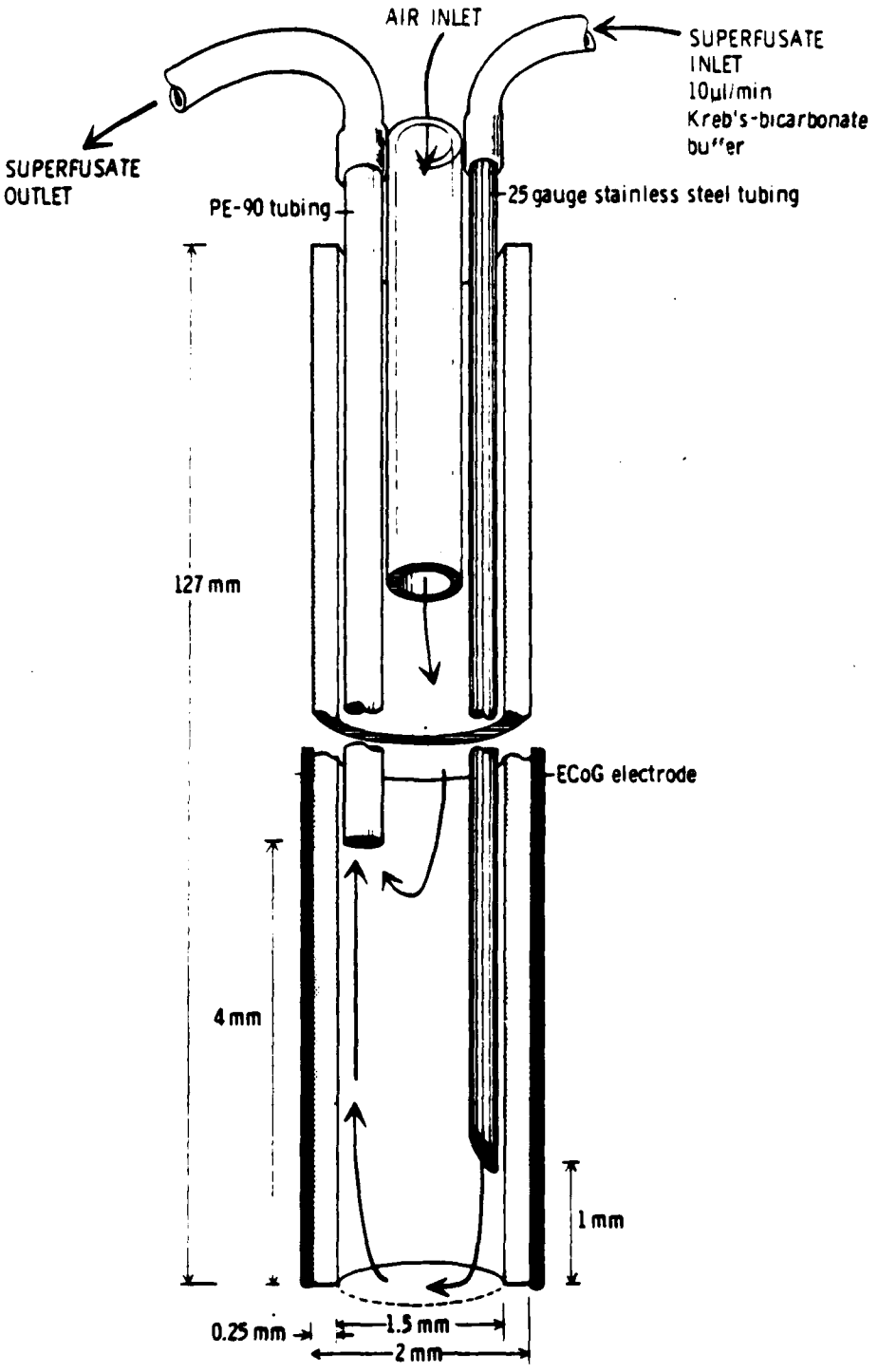
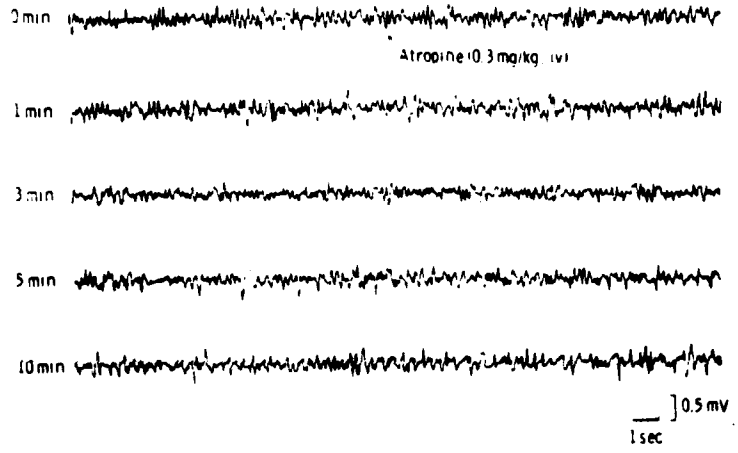


Figure I

A schematic of the device used to apply atropine to the cortical surface and to collect samples for measuring the release of ACh following fastigial nucleus stimulation.



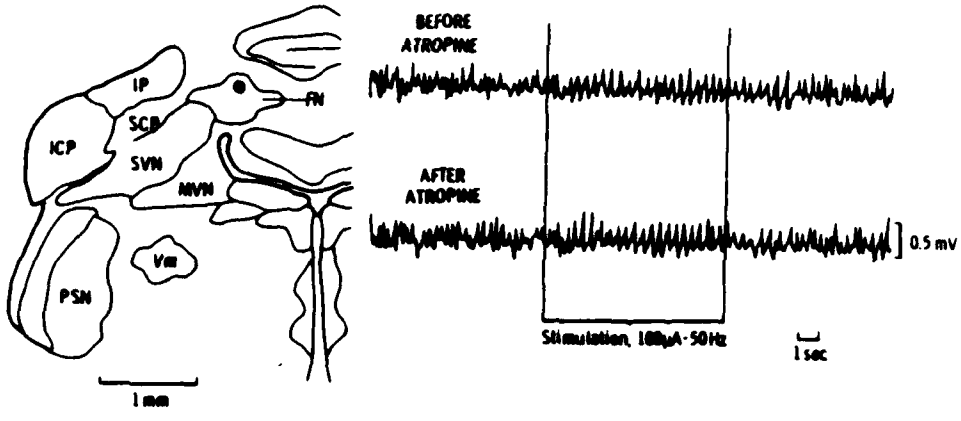


Figure II

Effect of administration of atropine sulfate (0.3 mg/kg, i.v.); on the EEG changes produced by electrical stimulation of the cerebellar *fastigial* nucleus in anesthetized rat (panel A) and on basal EEG activity (panel B).

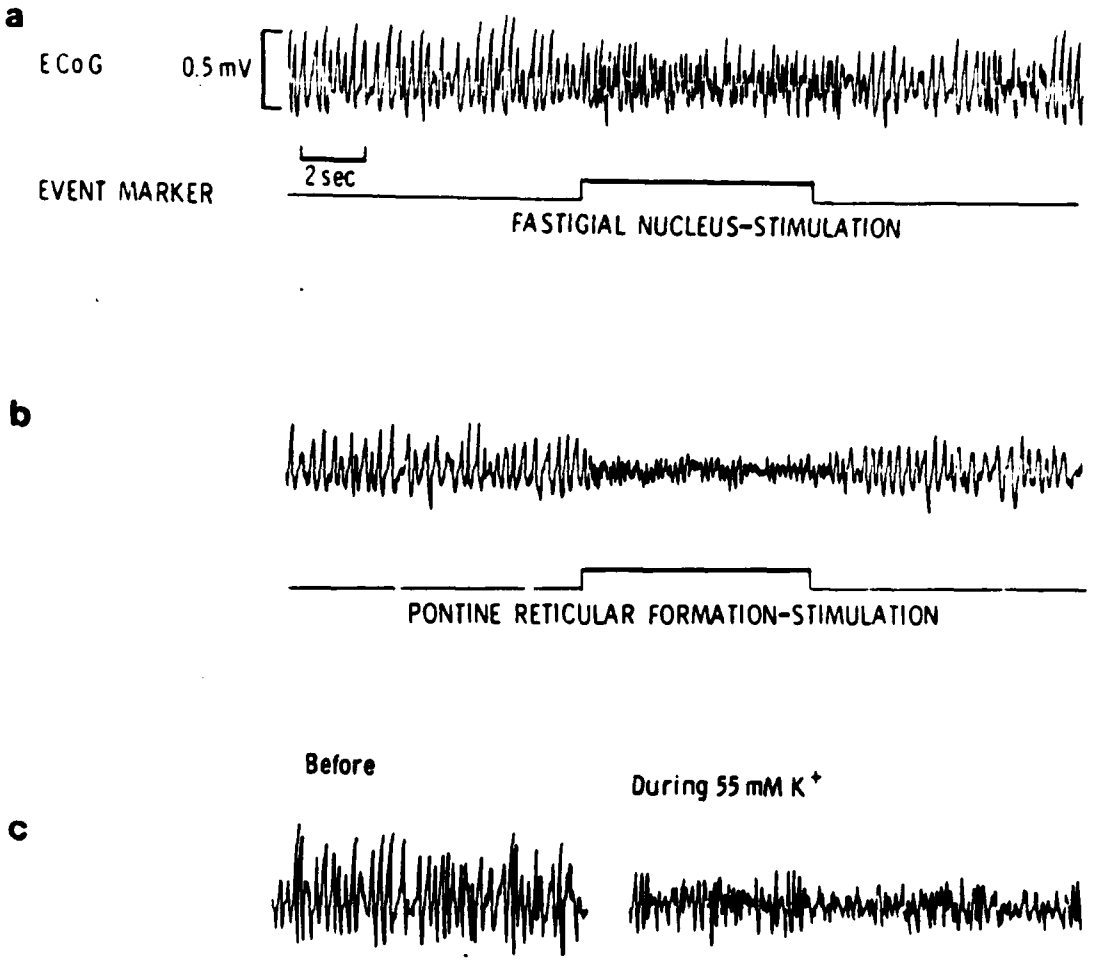


Figure III

Representative electrocorticograms (ECoG) recorded from the brain tissue beneath the cortical superfusion device; position on anesthetized rats during: (panel A) - electrical stimulation of fastigial nucleus (50 Hz, 100 uA); (panel B) -electrical stimulation of pontine reticular formation (50 Hz, 100 uA); potassium depolarization of the cerebral cortex.

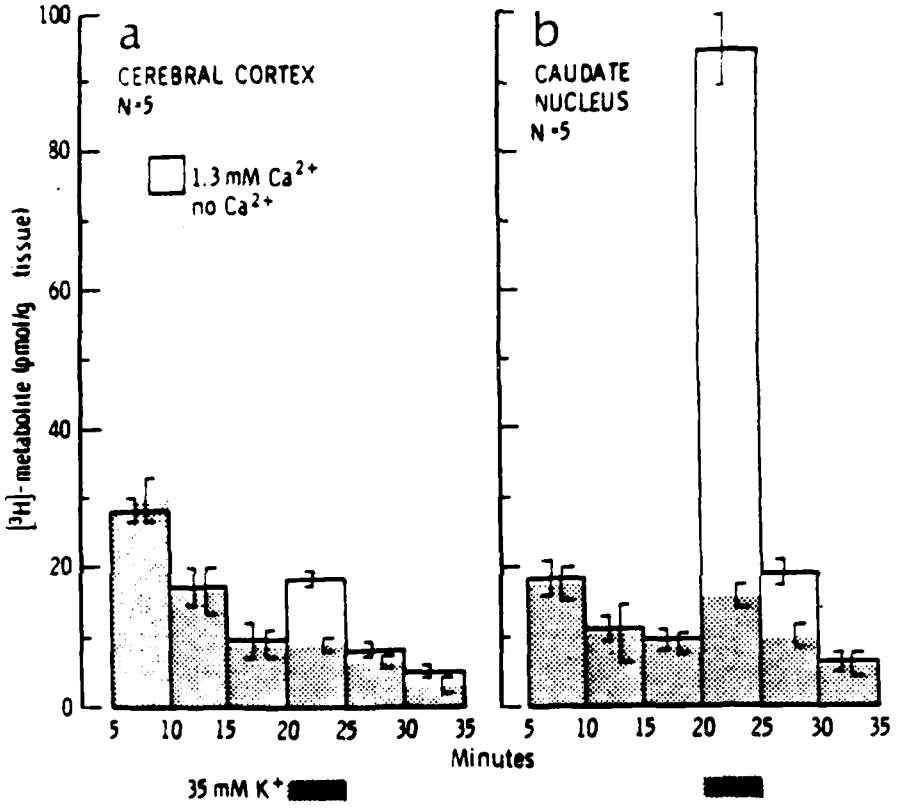


Figure IV

Effect of calcium on the basal and potassium-evoked release of ^{3}H-ACh from the cerebral cortex (Panel A) and caudate nucleus (Panel B).

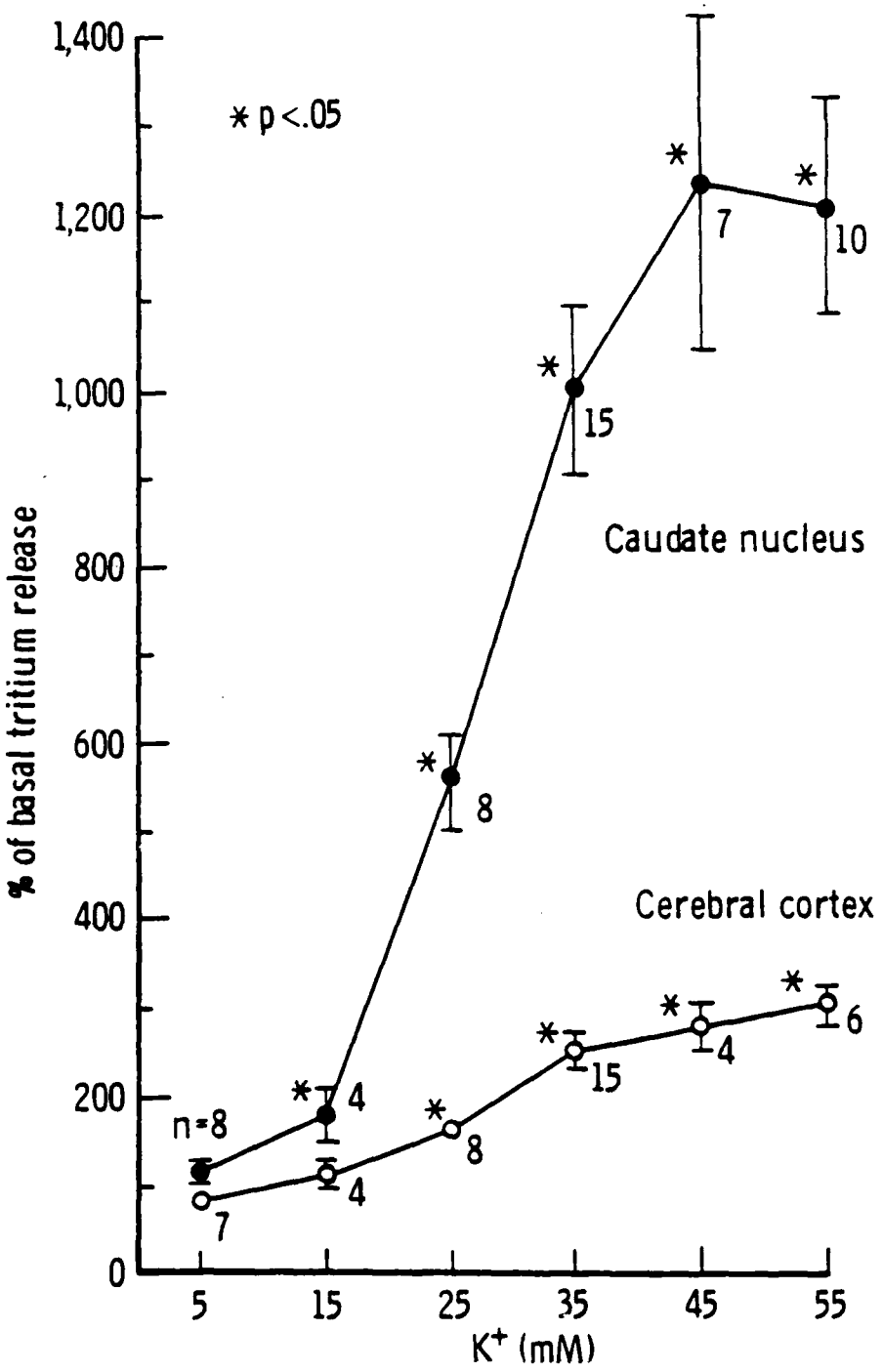


Figure V

Effect of increasing depolarization stimulus (i.e., potassium concentration) on the release of ^{3}H-ACh from brain slices prepared from the cerebral cortex and, for comparison, the caudate nucleus.

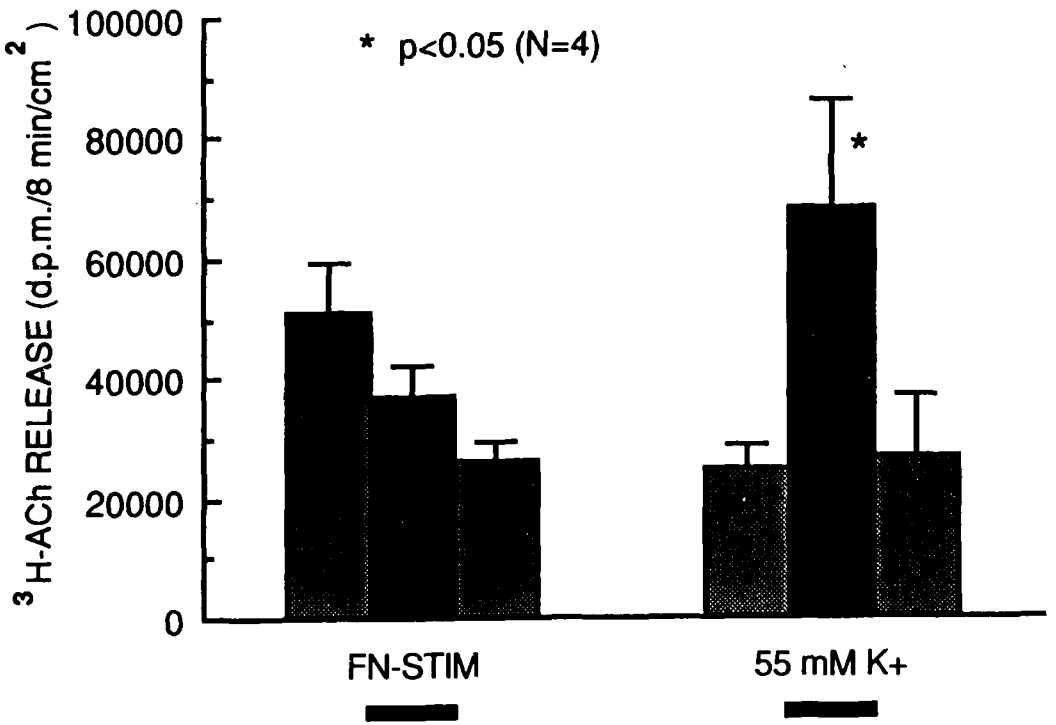


Figure VI

Effects of electrical stimulation of the fastigial nucleus (FN) and of potassium depolarization on the release of ^{3}H-ACh from the cortex of anesthetized rat.

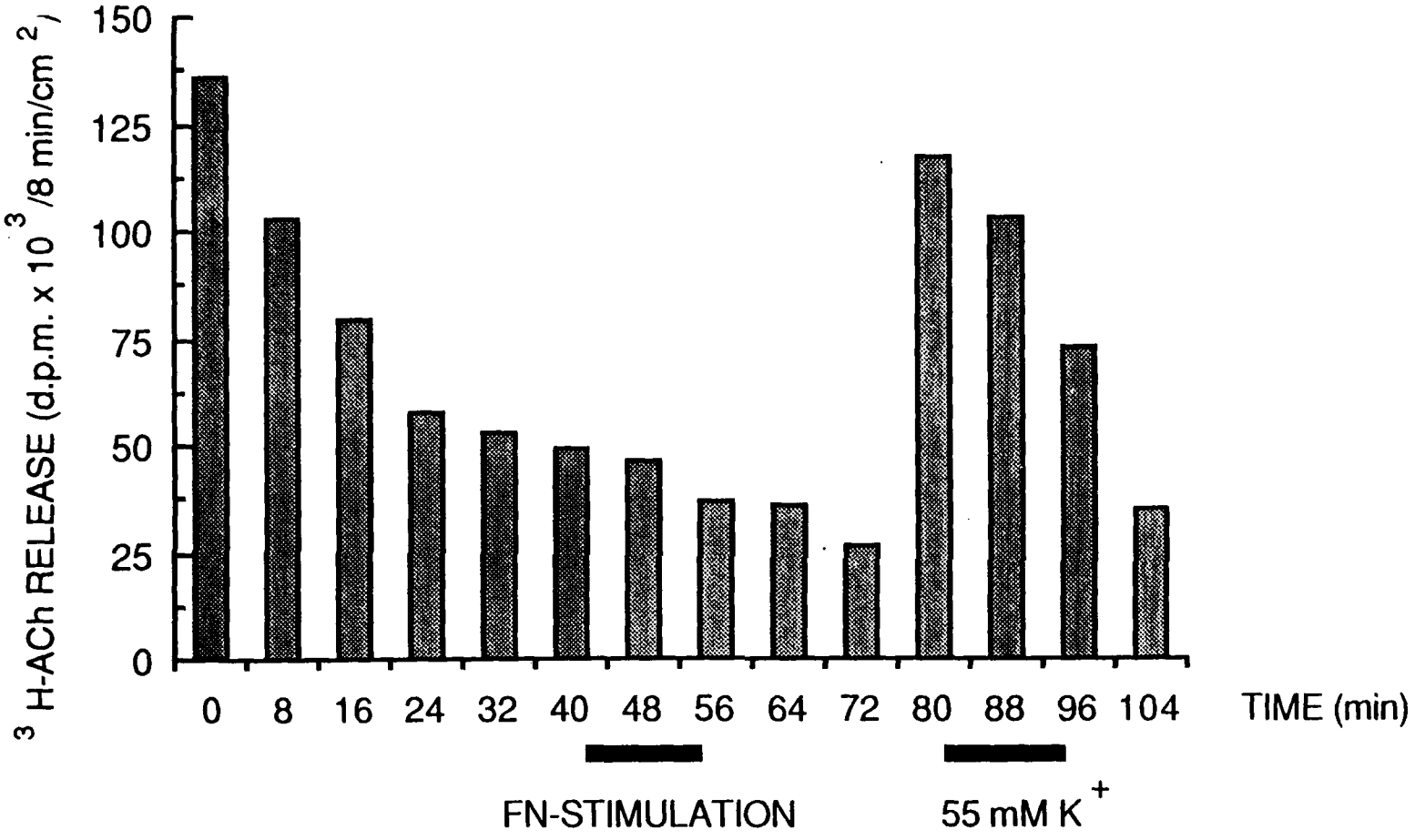


Figure VII

A representative experiment showing the effects of electrical stimulation of the fastigial nucleus (FN) and of potassium depolarization on the release of ^{3}H-ACh from the parietal cortex of anesthetized rat.

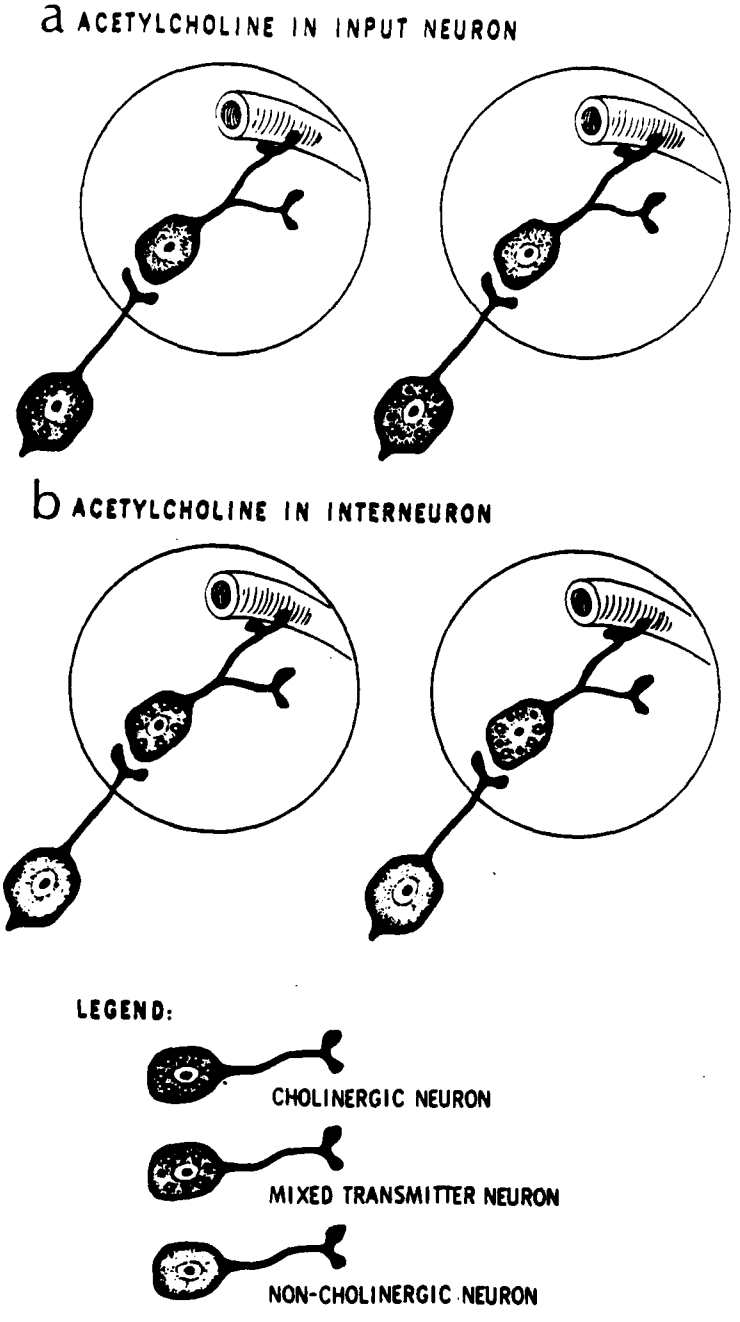


Figure VIII

A schematic showing the two most probable sources of cholinergic neurons innervating the cerebral vasculature.

	Left		Right	
	dpm/mg	% Total	dpm/mg	% Total
Frontal Cortex	5 ± 3	0.02	51 ± 16	0.22
Parietal Cortex	10 ± 8	0.04	**22801 ± 3266**	**99.16**
Occipital Cortex	15 ± 4	0.07	53 ± 12	0.04
Caudate Nucleus	5 ± 3	0.02	33 ± 22	0.14
Hippocampus	8 ± 3	0.03	12 ± 5	0.05

N=5

Table I

Disposition of radioactivity in brain regions one hour following microinjection of ^{3}H-choline into the right parietal cortex.

	Before Atropine			After Atropine		
	Resting	FN-Stim (a)	Δ	Resting	FN-Stim (a)	Δ
AP (mmHg)	128 ± 4	206 ± 7*	78	131 ± 6	211 ± 10*	80
HR (bpm)	430 ± 8	529 ± 2*	99	505 ± 6#	539 ± 7*	34#
Theshold current (uA)	-	21 ± 5		-	20 ± 2	

(a) FN-Stim at 50 Hz and 5 x threshold current
* $p < 0.001$ (paired t-test), from resting before atropine
# $p < 0.005$ from corresponding Δ before atropine
N=5

Table II

Effect of atropine sulfate (0.3 mg/kg i.v.) on the minimal current required to elevate arterial pressure (AP) (threshold current) (mean ± S.E.M.) and on the elevation in AP and heart rate (HR) produced by stimulation of the fastigial nucleus (FN-stim) in anesthetized rat.

Region	rCBF (ml/100 g x min)					
	Unstimulated (a)		FN-Stimulation			
	Untreated	Atropine	Untreated	% of Cont.	Atropine	% of Cont.
Medulla	80 ± 4	83 ± 7	127 ± 11	159**	92 ± 3	111
Cerebellum	84 ± 8	92 ± 6	110 ± 5	131*	104 ± 4	113
Pons	91 ± 5	85 ± 7	123 ± 9	152**	98 ± 4	115
Inferior Colliculus	111 ± 9	117 ± 8	135 ± 9	122	124 ± 5	106
Superior Colliculus	106 ± 7	104 ± 9	130 ± 10	123*	105 ± 4	101
Hypothalamus	98 ± 4	98 ± 10	119 ± 7	121*	97 ± 3	99
Thalamus	86 ± 1	90 ± 9	141 ± 15	164**	103 ± 6	114
Hippocampus	77 ± 5	79 ± 7	108 ± 12	140*	82 ± 5	104
Caudate n.	84 ± 4	89 ± 8	127 ± 16	151**	90 ± 7	101
Frontal cortex	84 ± 4	99 ± 9	207 ± 42	246**	105 ± 9	106
Parietal cortex	88 ± 6	108 ± 9	182 ± 32	207**	120 ± 10	111
Occipital cortex	79 ± 6	95 ± 8	127 ± 20	161*	110 ± 15	116
Corpus callosum	54 ± 3	61 ± 6	82 ± 9	152*	61 ± 4	100

* $p < 0.05$, ** $p < 0.01$; analysis of variance and Newman-Keuls test
(a) Differences between groups not significant ($p > 0.05$)

Table III

Effect of stimulation of the fastigial nucleus (FN) on rCBF (mean ± S.E.M.) with and without administration of atropine (0.3 mg/kg, i.v.).

	Unstimulated		FN-Stimulation	
	Untreated	Atropine	Untreated	Atropine
MAP (mmHg)	121 ± 4	122 ± 6	134 ± 3.2	127 ± 2
pCO_2 (mmHg)	36.6 ± 0.5	36 ± 1.2	35.9 ± 0.8	34.3 ± 1
pO_2 (mmHg)	369 ± 27	330 ± 31	390 ± 30	385 ± 28
pH	7.43 ± 0.02	7.41 ± 0.02	7.34 ± 0.04	7.37 ± 0.01
N	5	5	5	5

Differences among groups not significant ($p > 0.05$; analysis of variance and Newman-Keuls test).

Table IV

Mean arterial pressure (MAP) and blood gases (mean ± S.E.M.) in rats with and without electrical stimulation of the fastigial nucleus (FN) or administration of atropine.

Brain Region		lCBF (ml/100 g x min) Unstimulated(a)	FN-Stimulation(a)(b)	% of Control
Cerebral Cortex				
Frontal	R	139 ± 3	246 ± 27	177
	L	134 ± 5	244 ± 38	183
Sensorimotor	R	132 ± 14	252 ± 9	191
	L	126 ± 9	232 ± 6	185
Parietal	R	138 ± 30	232 ± 10	168
	L	139 ± 27	216 ± 9	154
Auditory	R	142 ± 42	229 ± 36	161
	L	128 ± 25	222 ± 28	173
Visual	R	119 ± 18	186 ± 9	156
	L	119 ± 18	176 ± 4	148
Hippocampus	R	106 ± 8	205 ± 23	194
	L	110 ± 9	202 ± 11	183
Basal Ganglia				
Caudate-Putamen	R	111 ± 16	187 ± 24	169
	L	111 ± 18	175 ± 12	158
Amygdala	R	102 ± 7	134 ± 19	131
	L	99 ± 6	149 ± 30	151
Thalamic Nuclei				
Anterior	R	125 ± 19	254 ± 22	203
	L	118 ± 14	233 ± 3	197
Reticular	R	85 ± 18	158 ± 12	186
	L	89 ± 18	167 ± 8	188
Ventral	R	102 ± 10	177 ± 31	173
	L	103 ± 11	176 ± 13	171
Intralaminar		110 ± 18	214 ± 23	194
Cerebellum				
Vermis		103 ± 10	180 ± 16	174
Hemisphere	R	91 ± 8	122 ± 5	135
	L	95 ± 7	159 ± 11	168
Dentate n.	R	180 ± 4	266 ± 13	148
	L	171 ± 10	317 ± 27	185
Hypothalamus	R	97 ± 7	151 ± 28	157
	L	99 ± 9	150 ± 28	152

Table V

Effect of stimulation of the fastigial nucleus (FN) on lCBF (mean ± S.E.M.) in anesthetized paralyzed rat.

Pons				
Vestibular comp.	R	189 ± 15	346 ± 29	183
	L	186 ± 10	365 ± 30	196
Parabrachial comp.	R	114 ± 9	202 ± 26	177
	L	107 ± 7	199 ± 28	185
Medullary Reticular N.				
Gigantocellular	R	107 ± 6	208 ± 2	195
	L	104 ± 4	210 ± 6	203
Parvocellular	R	105 ± 6	207 ± 9	198
	L	98 ± 2	224 ± 18	229
Corpus Callosum	R	57 ± 15	95 ± 14	166
	L	61 ± 14	99 ± 9	161

Values are means ± S.E.M. (N=3-5)

(a) No right-to-left differences (paired t-test, $p > 0.05$)

(b) Values are significantly elevated above those in unstimulated control ($p < 0.05$)

Table V

Effect of stimulation of the fastigial nucleus (FN) on lCBF (mean ± S.E.M.) in anesthetized paralyzed rat (continued).

REGION	rCBF (ml/100 g x min)				
	UNSTIMULATED (a)		FN-STIMULATION		
	Intact	Adrn.	Intact	Adrn.	Methylatrop. Adrn.(b)
Medulla	84 ± 4	80 ± 4	119 ± 10**	127 ± 11**	87 ± 3
Pons	88 ± 4	81 ± 5	127 ± 11**	123 ± 9**	86 ± 2
Thalamus	87 ± 4	86 ± 1	165 ± 13**	141 ± 15**	92 ± 8
Caudate n.	87 ± 1	84 ± 4	131 ± 7**	127 ± 16**	90 ± 6
Frontal Cortex	98 ± 5	84 ± 4	192 ± 7**	207 ± 42**	81 ± 9
Parietal Cortex	103 ± 4	88 ± 6	192 ± 6**	182 ± 32**	101 ± 8
N	5	5	5	5	5

**p < 0.01 from respective control (analysis of variance and Newman-Keuls test)
(a) Differences between groups not significant (p > 0.05)
(b) p > 0.05 with respect to unstimulated group

Table VI

Effect of stimulation of the fastigial nucleus (FN) on rCBF (mean ± S.E.M.) with and without adrenalectomy (Adrn.) and administration of methylatropine (Methylatrop., 0.3 mg/kg, i.v.).

	Unoperated Control		Parietal Craniotomy			
			(Vehicle)	(Vehicle)	(Vehicle)	(Atropine)
	L	R	L	R	L	R
Frontal Cortex	92 ± 8	94 ± 9	93 ± 12	79 ± 7	100 ± 13	91 ± 13
Parietal Cortex	95 ± 7	93 ± 5	82 ± 12	70 ± 6	83 ± 7	85 ± 9
Occipital Cortex	87 ± 8	89 ± 7	86 ± 12	72 ± 7	88 ± 6	85 ± 9
Caudate Nucleus	92 ± 14	82 ± 6	75 ± 6	75 ± 6	84 ± 7	85 ± 10
Hippocampus	70 ± 5	73 ± 5	78 ± 8	74 ± 6	72 ± 3	78 ± 7
N	6	6	6	6	4	4

rCBF expressed as ml/100 g/min

Data are means ± S.E.M.

Table VII

Effect of parietal craniotomy and atropine sulfate (100 uM) on resting regional cerebral blood flow.

	UNSTIMULATED		FN-STIMULATION		HYPERCARBIA
	VEHICLE (N=6)	ATROPINE (N=4)	VEHICLE (N=11)	ATROPINE (N=5)	ATROPINE (N=5)
MAP (mmHg)	124 ± 6	136 ± 4	134 ± 2	130 ± 5	110 ± 6
pO_2 (mmHg)	429 ± 19	431 ± 20	427 ± 10	409 ± 21	408 ± 30
pCO_2 (mmHg)	35.7 ± 0.5	34.9 ± 1.0	36.5 ± 0.5	36.6 ± 0.9	59.0 ± 1.4*
pH	7.41 ± 0.02	7.41 ± 0.01	7.32 ± 0.02*	7.32 ± 0.01*	7.24 ± 0.01*

Values represent means ± S.E.M.; animals were anesthetized, paralyzed and artificially respirated.

*$p < 0.05$, significantly different from vehicle control.

Table VIII

Mean arterial pressure (MAP), pH and arterial blood gases in rats examined.

		UNSTIMULATED	FN-STIMULATION		HYPERCARBIA
		VEHICLE (N=6)	VEHICLE (N=11)	ATROPINE (d) (N=5)	ATROPINE (d) (N=5)
pH	R	7.40 ± 0.05	7.38 ± 0.05	7.54 ± 0.05	7.63 ± 0.09
	L	7.46 ± 0.80	7.35 ± 0.04	7.39 ± 0.07	7.58 ± 0.09
pCO_2 (mmHg)	R	32.7 ± 4.2	31.9 ± 1.8	25.8 ± 6.0	21.8 ± 3.1*
	L	33.7 ± 3.8	31.8 ± 1.6	32.9 = 5.1	20.5 ± 2.0*
pO_2 (mmHg)	R	428 ± 77	414 ± 49	394 ± 67	373 ± 72
	L	425 ± 78	410 ± 49	463 ± 77	384 ± 50

*$p < 0.05$, significantly different from corresponding vehicle, unstimulated controls (ANOVA).

(a) Values are means ± S.E.M.

(b) No right-to-left differences with any treatment ($p > 0.05$; paired t-test).

(c) Kreb's-bicarbonate buffer solutions were bubbled with 95% O_2: 5% CO_2, the above values determined and the solution applied to the parietal cortex. Solutions were continuously bubbled with 95% O_2: 5% CO_2 following application to the cortex.

(d) Atropine sulfate (100 uM) applied to right parietal cortex.

Table IX

pH, PCO_2 and pO_2 of buffer solutions applied to the parietal cortex of rats with and without FN-stimulation, or with hypercarbia.

Brain Region		rCBF (ml/100 g x min) + S.E.M. UNSTIMULATED VEHICLE(a) (N=6)	UNSTIMULATED ATROPINE(a) (N=4)	FN-STIMULATION VEHICLE(a)(c) (N=11)	FN-STIMULATION ATROPINE(a)(b)(c) (N=5)	HYPERCARBIA ATROPINE(a)(b)(c) (N=5)
Frontal Cortex	R	79 ± 7	91 ± 13	206 ± 14	**133 ± 30***	200 ± 42
	L	93 ± 12	100 ± 13	195 ± 9	160 ± 23	188 ± 31
Parietal Cortex	R	70 ± 6	85 ± 9	180 ± 13	**129 ± 22***	168 ± 26
	L	82 ± 12	83 ± 7	188 ± 16	165 ± 24	171 ± 28
Occipital Cortex	R	72 ± 7	85 ± 9	168 ± 11	130 ± 22	184 ± 34
	L	86 ± 12	88 ± 6	167 ± 7	148 ± 20	155 ± 25
Caudate Nucleus	R	75 ± 6	85 ± 10	157 ± 8	128 ± 16	174 ± 7
	L	75 ± 6	84 ± 7	155 ± 5	137 ± 15	176 ± 7
Hippocampus	R	74 ± 6	78 ± 7	126 ± 5	103 ± 7	172 ± 15
	L	78 ± 8	72 ± 3	130 ± 6	111 ± 9	168 ± 8

(a) Differences between right and left sides not significant ($p > 0.05$; paired t-test).
(b) Atropine sulfate (100 uM) applied to only right parietal cortex.
(c) All values significantly increased above those in unstimulated vehicle control ($p < 0.05$; ANOVA).
* Values significantly decreased from those in corresponding vehicle control, FN-stimulated group ($p < 0.05$; ANOVA).

Table X

Effects of topical application of atropine sulfate on regional cerebral blood flow (rCBF) in rats with and without stimulation of fastigial nucleus (FN) or with hypercarbia.

	Fractional Release of Tritium (% of Total d.p.m./min/mg tissue) (a)			
	Caudate Nucleus		Cerebral Cortex	
Treatment	basal	K^+	basal	K^+
with Ca^{2+} (1.2 mM)	0.67 ± 0.10	6.31 ± 0.70 *	1.85 ± 0.29	3.38 ± 0.50 *
without Ca^{2+} (0.0 mM)	0.50 ± 0.02	0.62 ± 0.04	1.28 ± 0.27	1.16 ± 0.22

(a) Tissue slices of rat caudate nucleus and cerebral cortex were incubated in Kreb's-bicarbonate buffer containing ^3H-choline (1 uM; 20 uCi/ml) at 37°C for 30 min, then transferred through test tubes with isotope-free buffer which had the indicated concentrations of calcium and potassium added. Values are the mean ± S.E.M.

* Indicates the value is statistically different from the basal release; N=3.

Basal release refers to the release obtained for the 5 min period immediately prior to K^+ depolarization.

Table XI

Effect of extracellular calcium and depolarization with 35 mM K^+ on the release of ACh from slices of rat caudate nucleus and cerebral cortex.

DISCUSSION AND CONCLUSIONS

The two objectives set forth for year 01 of the contract have, in large part, been completed. First, we have replicated the well-established increase in rCBF elicited by FN-stimulation and established that systemic atropine administration completely blocks this cerebrovasodilation. This finding indicates that ACh is released at some link along the neural pathway from the cerebellar FN to the cerebrovasculature. Second, we have demonstrated that the FN-elicited increase in cortical rCBF, an effect that occurs independently of changes in cerebral glucose utilization, is attenuated by local application of atropine to the surface of the parietal cortex. These data suggest that the cortical cerebrovasodilation elicited by FN-stimulation is mediated by the release of ACh at the level of the cerebral cortex. Another essential line of evidence that a cholinergic mechanism is in the cerebral cortex is critical for the FN-vasodilation would be to demonstrate that ACh is released locally with FN-stimulation. Our data indicate that the amount of ACh released locally in the parietal cortex following FN-stimulation is too small to be detected by the currently available assay techniques. Additional discussion of these findings is given below.

Effects of Systemic Atropine on FN-elicited Cerebrovasodilation

The study using systemic atropine administration demonstrates that blockade of muscarinic cholinergic receptors by atropine abolished the well-established increase in rCBF elicited by FN-stimulation. Since atropine did not modify the associated elevation in AP or slowing of the EEG, the cholinergic blockade is relatively selective, affecting only the cerebrovascular component of the responses to excitation of FN. The blockade by atropine of the cerebrovascular vasodilation elicited from FN cannot be attributed to effects of the drug upon gases or AP, since these parameters did not differ among experimental groups. Nor is it likely to be a consequence of a non-specific effect of the drug upon cerebral circulation, since atropine had no effect on resting rCBF and the vasodilation elicited by stimulation of the dorsal medullary reticular formation in rat is not affected by the drug (20). It is also unlikely that atropine's effect upon the cerebrovascular response is mediated by blockade of muscarinic receptors located outside the CNS, since the vasodilation elicited by FN-stimulation occurs through neural pathways entirely contained within brain (6, 9). In particular, its action cannot be attributed to blockade of the purported cholinergic pathway innervating the cerebral vasculature through the facial and greater superficial petrosal nerves (1, 21), since, in rabbit, transection of this nerve does not affect the vasodilation elicited from FN (7).

Of special interest was the observation that systemic administration of methylatropine, a muscarinic receptor antagonist that weakly crosses the blood-brain barrier, also blocked the FN-elicited increase in rCBF. It is unlikely that methylatropine passed to deeper-lying structures that may participate in the FN-elicited cerebrovasodilation because of the physico-chemical nature of methylatropine. Instead, we interpret this finding to suggest that the cholinergic link may lie in close apposition to cerebral blood vessels that could conceivably be exposed to sufficiently high concentrations of methylatropine.

It is therefore likely that the global abolition of the vasodilation elicited from FN is due to blockade of central cholinergic receptors of the muscarinic type contained within brain and located on cerebral vessels and/or upon neurons situated near cerebral vessels. In vessels, muscarinic receptors are localized to all segments of cerebral vasculature, including large cerebral arteries (22), small pial arteries (23) and capillaries (24), wherein the receptors are associated with endothelial and/or smooth muscle cells (25). Neuronal muscarinic receptors have a heterogeneous distribution throughout the brain, with highest concentrations in cerebral cortices (mostly laminae I, III, VI), caudate-putamen, hippocampus, and cranial nerve nuclei (26, 27).

However, if the blockade is at the vascular level, the finding would suggest that the ultimate neuro-vascular effector mediating the vasodilation is cholinergic. Supporting this possibility are the facts that ACh dilates cerebral arteries in vitro (22), or in vivo (4), and intracarotid infusion (4) or topical application of ACh (23) increases CBF. These effects are attenuated by atropine.

Effects of Cortically Applied Atropine on FN-elicited Cerebrovasodilation

The local application study indicates that the cholinergic link in the FN-elicited increase in cortical rCBF may reside locally in the cortex, perhaps within, or in close apposition to, the the cortical blood vessels. The following evidence supports this conclusion. First, atropine applied to the right parietal cortex attenuated by 55% the vasodilation elicited by FN-stimulation, while adjacent structures were unaffected. Second, the effect of atropine was not due to vasoparalysis, since atropine did not modify the vasodilation elicited by CO_2.

The finding that cortical application of atropine prevented only 55% of the cortical dilation contrasts like the fact that atropine given systemically reduced the vasodilation by 92%. One likely possibility for the discrepancy between the two studies is that the area of the parietal cortex removed for analysis is much larger than the area affected by application of atropine, and this dilution effect would underestimate the ability of atropine to block the vasodilation. On the other hand, a co-transmitter may be required for the dilation to occur. Although less likely, this possibility is consistent with the recent report that ACh and vasoactive intestinal polypeptide, another potent vasodilator (4), co-exist in a subpopulation of local cortical neurons (13). Additional experiments using microinjection techniques for atropine administration and autoradiography to measure local cortical blood flow will be required to resolve these possibilities.

Cortical Release of ACh Following FN-stimulation

In support of the concept that ACh could be released onto cortical blood vessels following FN-stimulation is the fact that elements within, or in close apposition to, these vessels can synthesize ACh. Others have shown activity of choline acetyltransferase (ChAT) in close association with cerebral blood vessels (25). In related studies performed in this laboratory, we have found that authentic ACh is synthesized by cortical microvessels, and, moreover, that the vessels contain a specific uptake mechanism for choline, the precursor to ACh. The absolute amount of ACh synthesized within the cortex by elements associated with microvessels is relatively small, since cortical microvessels constitute a small portion of the total brain tissue (2-4%, based on protein contents). However, based on specific activities of ChAT, microvessels have the remarkable capacity to synthesize ACh at rates equivalent to those of synaptosomes (28). This finding suggests that concentration of ACh at the neurovascular junction may be as high as in the transneuronal site.

However, in the in vivo release experiments, we did not detect the stimulus-locked release of ACh during FN-stimulation. This lack of effect was not the result of our inability to accurately increase the release of ACh from the cortex, since potassium depolarization resulted in a marked stimulus-locked release of ACh. Rather, it is possible that since such a small population of cortical cholinergic activity is associated with cerebral blood vessels, there was too little ACh release during FN-stimulation to be detected by currently available techniques.

The source of ACh released within the cortex following FN-stimulation and the neural pathway from FN to cortex are unknown. Neuroanatomical tracing studies show that projections from FN do not reach the cortex directly (8). Therefore, the pathway from FN to the cortex is polysynaptic. The two most likely sources for the cholinergic link in this pathway are local cholinergic neurons of the cortex, or nerve terminals of afferent cholinergic fibers arising from or passing through the basal forebrain (12).

While lesions of the basal forebrain (12). While lesions of the basal forebrain and selective removal for intrinsic cortical neurons (29) both reduce cortical ChAT activity and prevent the cortical vasodilation elicited by FN-stimulation, these experiments still do not establish whether the source of the ACh is in afferent or local neurons. Studies are in progress to delineate which source of ACh mediates the FN-elicited cortical vasodilation. See Fig. VIII for a schematic of these two possibilities.

Objectives for Year 02
We will seek to establish:

1. Whether the ACh released in cortex by FN-stimulation is from local neurons or input pathways

2. Whether local injection of cholinergic agonists will increase local CBF and whether cholinesterase inhibitors will increase local CBF.

3. Whether local injection of cholinesterase inhibitors will alter local cerebral glucose utilization

4. Whether local injection of cholinesterase inhibitors will facilitate the cholinergically indicated vasodilation.

We have recently made arrangements to begin the contracted work with nerve agents. Using diisopropylfluorophosphonate (DFP) as a prototype, we hope to begin the initial studies during the first quarter to determine the effects on CBF of nerve agents microinjected into the cortex.

Importance of Future Objectives: Military Significance
The demonstration that organophosphates will facilitate the vasodilation produced by intrinsic cholinergic systems in the brain predicts that the agent will alter the cerebrovascular autoregulation and that this in turn will result in vulnerability of the blood-brain barrier when blood pressure varies in the upper or lower range of autoregulation. By suspending autoregulation, the brain becomes vulnerable to fluctuations of systemic arterial pressure, which under normal conditions will not have any deleterious effects. Thus, reduction of arterial pressure as a consequence of trauma, or dehydration in the soldier exposed to organophosphates, will result in a great vulnerability of the brain to cerebral ischemia and possibly infarction. Conversely, the abolition of autoregulation will make the brain more vulnerable to elevations of AP, such as might be experienced by emotional or physical stress, leading thereby to breakdown of the blood-brain barrier, edema, and even hemorrhage. The recognition that organophosphates will alter autoregulation will indicate the importance of maintaining the AP within normal range in exposed persons, and may help to avert, in some circumstances, cerebrovascular consequences of organophosphate poisons.

In addition, an understanding of the action of organophosphates on cerebral cholinergic control may help to explain some of the mental changes associated with impairment of central cholinergic systems, i.e. that they are acting through control of local circulation rather than by direct effects on neural transmission.

LITERATURE CITED

1. Bevan, J.A., Buga, G.M., Jope, C.A., Jope, R.S. and Moritoki, H. Further evidence for a muscarinic component to the neural vasodilator innervation of cerebral and cranial extracerebral arteries of the cat. Circ. Res. 51:421-429, 1982.

2. Reis, D.J. Nervous control of the cerebral blood flow in normal health and in relationship to cerebrovascular disease. In: Maladies Vasculaires Cerebrales: Cerebrovascular Diseases. Deuxieme Conferences de la Salpetriere, P. Castaigne, F. L'hermitte,J.-C. Gautier, and A. Moriniere (eds.) J.-B. Baillier, Paris, 1979, pp. 197-224.

3. Reis, D.J., Iadecola, C. and Nakai, M. Control of cerebral blood flow and metabolism by intrinsic neural systems in brain. In: F. Plum and W. Pulsinelli, (Eds.), Cerebrovascular Diseases, Raven Press, New York, 1985, pp. 1-25.

4. Heistad, D.D., Marcus, M.L., Said, S.I. and Gross, P.M. Effect of acetylcholine and vasoactive intestinal polypeptide on cerebral blood flow. Am. J. Physiol. 239:H73-H80, 1980.

5. Pepeu, G. The release of acetylcholine from the brain: an approach to the study of the central cholinergic mechanisms. Prog. Neurobiol. 3:259-288, 1973.

6. Nakai, M., Iadecola, C., Ruggiero, D.A., Tucker, L.W. and Reis D.J. Electrical stimulation of cerebellar fastigial nucleus increases cerebral cortical blood flow without change in local metabolism:Evidence for an intrinsic system in brain for primary vasodilation. Brain Res. 260:35-49, 1983.

7. Reis, D.J., Iadecola, C., MacKenzie, E., Mori, M., Nakai, M. and Tucker, L., Primary and metabolically coupled cerebrovascular dilation elicited by stimulation of two intrinsic systems in brain. In: D. Heistad and M. Marcus (Eds.), Cerebral Blood Flow: Effects of Nerves and Neurotransmitters, Elsevier/North Holland, New York, 1982, pp. 475-484.

8. Del Bo, A., Ruggiero, D.A., Ross, C.A., Wiley, R. and Reis, D.J. Pathways from the fastigal pressor area in rat. Soc. Neurosci. Abstr. 8: 76, 1982.

9. Nakai, M., Iadecola, C., and Reis, D.J. Global cerebral vasodilation elicited by stimulation of rat fastigial cerebellar nucleus. Am. J. Physiol. (Heart & Circ. Physiol.) 243:H226-H235, 1982.

10. Iadecola, C., Mraovitch, S., Meeley, M.P. and Reis, D.J. Do cholinergic neurons of the basal forebrain cholinergic system mediate the cortical vasodilation elicited by fastigial nucleus stimulation in rat ? J. Cereb. Blood Flow Metab. 3:S178-S179, 1983.

11. Lehman, J. Nagy, J.I., Atmadja, S. and Fibiger, H.C. The nucleus basalis magnocellularis: the origin of a cholinergic projection to the neocortex of the rat. Neuroscience, 5:1161-1174, 1980.

12. Fibiger, H.C. The organization and some projections of cholinergic neurons of the mammalian forebrain. Brain Res. Rev., 4:327-388, 1982.

13. Eckenstein, F. and Baughman, R.W. Two types of cholinergic innervation in cortex, one co-localized with vasoactive intestinal polypeptide. Nature 309:153-155, 1984.

14. Ohno, K., Pettigrew, K.D. and Rappoport, S.I. Local cerebral blood flow in the conscious rat as measured with ^{14}C-iodoantipyrine and ^{3}H-nicotine. Stroke 10:62-67, 1979.

15. Hernandez, M.J., Brennan, R.W. and Bowman, G.S. Cerebral blood flow autoregulation in the rat. Stroke, 9:150-155, 1978.

16. Hadhazy, P. and Szerb, J.C. The effect of cholinergic drugs on ^{3}H-acetylcholine release from slices of rat hippocampus, striatum and cortex. Brain Res. 123:311-322, 1977.

17. Briggs, C.A. and Cooper, J.A. A synaptosomal preparation from the guinea pig ileum myenteric plexus. J. Neurochem. 36:1097-1108, 1981.

18. Richter, J.A. and Marchbanks, R.M. Synthesis of radioactive acetylcholine from (^{3}H)-choline and its release from cerebral cortex slices in vitro. J. Neurochem. 18:691-703, 1971.

19. Miura, M. and Reis, D.J. Cerebellum: A pressor response elicited from the fastigial nucleus and its efferent pathway in brainstem. Brain Res. 13:595-599, 1969.

20. Iadecola, C., Nakai, M., Mraovitch, S., Tucker, L. and Reis, D.J. Global increase in cerebral metabolism and blood flow during stimulation of a subnucleus of the medullary reticular formation. Neurology, 32(2):A111, 1982.

21. Vasquez, J. and Purves, M.J. The cholinergic pathways to cerebral blood vessels. I. Morphological studies. Pflugers Arch. 379: 157-163, 1979.

22. Lee, T.J.F., Hume, W.R., Su, C. and Bevan, J.A. Neurogenic vasodilation of cat cerebral arteries. Circ. Res. 42:535-542, 1978.

23. Kuschinsky, W., Wahl, M. and Neiss, A. Evidence for cholinergic dilatory receptors in pial arteries of cats. Pflugers Arch. 347:199-208, 1974.

24. Estrada, C. and Krause, D., Muscarinic cholinergic receptor sites in cerebral blood vessels. J. Pharmacol Exp. Ther. 221:85-90, 1982.

25. Estrada, C. and Krause, D.N. Biochemical evidence for cholinergic innervation of intracerebral blood vessels. Brain Res. 266:261-270, 1983.

26. Kuhar, M.J. and Yamamura, H.I. Localization of cholinergic muscarinic receptors in rat brain by light microscopic radioautography. Brain Res. 110:229-243, 1976.

27. Wamsley, J.K., Zarbin, M.A. and Kuhar, M.J. Distribution of muscarinic cholinergic high and low affinity against binding sites: A light microscopic autoradiographic study. Brain Res. Bull. 12:233-243, 1984.

28. Arneric, S.P., Iadecola, C., Honig, M.A., Underwood, M.D. and Reis, D.J., Local cholinergic mechanisms mediate cerebral cortical vasodilation elicited by

electrical stimulation of the fastigial nucleus. Acta Physiol. Scand. 127(Suppl. 552):70-73, 1986.

29. Johnston, M.V., McKinney, M. and Coyle, J.T. Evidence for a cholinergic projection to neocortex from neurons in basal forebrain. Proc. Natl. Acad. Sci. 76:5392-5396, 1979.

DISTRIBUTION LIST

1 copy	Commander US Army Medical Research and Development Command ATTN: SGRD-RMI-S Fort Detrick, Frederick, Maryland 21701-5012
5 copies	Commander US Army Medical Research and Development Command ATTN: SGRD-PLE Fort Detrick, Frederick, Maryland 21701-5012
12 copies	Defense Technical Information Center (DTIC) ATTN: DTIC-DDAC Cameron Station Alexandria, VA 22304-6145
1 copy	Dean School of Medicine Uniformed Services University of the Health Sciences 4301 Jones Bridge Road Bethesda, MD 20814-4799
1 copy	Commandant Academy of Health Sciences, US Army ATTN: AHS-CDM Fort Sam Houston, TX 78234-6100

END

12-87

DTIC